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# The HIV-1 Antisense Protein (ASP) induces CD8 T cell responses during chronic infection

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## Abstract

**Background:** CD8+ T cells recognize HIV-1 epitopes translated from a gene's primary reading frame (F1) and any one of its five alternative reading frames (ARFs) in the forward (F2, F3) or reverse (R1-3) directions. The 3' end of HIV-1's proviral coding strand contains a conserved sequence that is directly overlapping but antiparallel to the *env* gene (ARF R2) and encodes for a putative antisense HIV-1 protein called ASP. ASP expression has been demonstrated *in vitro* using HIV-transfected cell lines or infected cells. Although antibodies to ASP were previously detected in patient sera, T cell recognition of ASP-derived epitopes has not been evaluated. We therefore investigated the *ex vivo* and *in vitro* induction of ASP-specific T cell responses as a measure of immune recognition and protein expression during HIV-1 infection.

**Results:** A panel of overlapping peptides was initially designed from the full-length ASP sequence to perform a global assessment of T cell responses. Recognition of ASP-derived antigens was evaluated in an IFN- $\gamma$ ELISpot assay using PBMCs from HIV-1 seropositive and seronegative individuals. Eight of 25 patients had positive responses to ASP antigens and none of the seronegative donors responded. As a complimentary approach, a second set of antigens was designed using HLA-I binding motifs and affinities. Two ASP-derived peptides with high predicted binding affinities for HLA-A\*02 (ASP-YL9) and HLA-B\*07 (ASP-TL10) were tested using PBMCs from HIV-1 seropositive and seronegative individuals who expressed the matching HLA-I-restricting allele. We found that HLA-I-restricted ASP peptides were only recognized by CD8+ T cells from patients with the relevant HLA-I and did not induce responses in any of the seronegative donors or patients who do not express the restrictive HLA alleles. Further, ASP-YL9-specific CD8+ T cells had functional profiles that were similar to a previously described HLA-A\*02-restricted epitope (Gag-SL9). Specific recognition of ASP-YL9 by CD8+ T cells was also demonstrated by tetramer staining using cells from an HLA-A\*02 HIV-infected patient.

**Conclusion:** Our results provide the first description of CD8+ T cell-mediated immune responses to ASP in HIV-1-infected patients, demonstrating that ASP is expressed during infection. Our identification of epitopes within ASP has implications for designing HIV vaccines.

**Keywords:** Chronic HIV-1, ASP, CD8+ T-lymphocytes, HLA-I, Cryptic epitopes, Alternative reading frame

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## Background

Thirty years after its appearance, the HIV/AIDS epidemic still represents a major public health threat worldwide. Strategies for developing effective host immunity and protection against HIV-1 infection or lifelong control of the virus are urgently needed. Multiple lines of evidence suggest that HIV-specific cytotoxic CD8<sup>+</sup> T cells (CTLs) participate in controlling viral replication [1-3]. During acute infection, the expansion of HIV-specific CTLs occurs prior to the appearance of neutralizing antibodies and is associated with decreased viremia [4,5]. Delayed disease progression has been correlated with the detection of Gag-specific CTLs and the presence of protective human leukocyte antigen class I (HLA-I) alleles, which effectively present HIV-1 derived epitopes for immune recognition [6-10]. However, HIV rapidly mutates to evade virus-specific T cell responses, underlying the selection pressure exerted by CTLs [11-13]. Thus, protection against HIV will likely require a vaccine that affords immediate CTL recognition of infected cells through sustained presentation of conserved T cell epitopes.

CTLs are stimulated by peptides presented by MHC-I molecules and generated from the proteolysis of all nine proteins encoded by the three forward reading frames of its sense mRNA strand. In addition to protein-derived epitopes, investigations over the last two decades have led to the discovery of cryptic epitopes, a novel set of T cell targets translated from alternative reading frames (ARFs) in both sense and antisense transcripts during autoimmune disease, cancer and several viral infections, including HIV-1 and SIV [14-26]. Moreover, sequence analysis of HIV-1 clade B isolates have repeatedly detected a conserved open reading frame in an ARF of *env*, suggesting the existence of a possible tenth viral protein [27-32]. Vaquero *et al.* demonstrated *in vitro* that this antisense ARF encodes a protein of 19kD, so called antisense protein (ASP) [33]. Subsequently, the existence of ASP has been supported by its *in vitro* expression from a native upstream promoter and, shortly thereafter, the detection of antibodies to ASP in patient sera [28,33,34]. Recently, we and others have confirmed that ASP is expressed by several cell types during HIV-1 infection [29,35-38]. Interestingly, ASP was detected in monocyte-derived macrophages and dendritic cells, which was consistent with preferential transcription of the antisense strand in these antigen-presenting cells (APC) [37]. Collectively, the aforementioned studies suggest that ASP is produced during the viral cycle. We previously demonstrated that ARF-derived HIV-1 antigens (i.e. cryptic epitopes, CE) are produced during infection by detecting CE-specific CTLs [17]. We therefore extended this work by evaluating T cell recognition of ASP-derived antigens in HIV-1 seropositive patients.

In an unbiased approach, we used pools of overlapping ARF peptides spanning the entire length of ASP to detect ASP-specific T cell responses in PBMCs obtained from HIV-1 seropositive and seronegative donors. We further investigated CD8<sup>+</sup> T cell responses to predicted HLA-I-restricted epitopes derived from ASP. Overall, our findings show that ASP is specifically targeted by CD8<sup>+</sup> T cells of chronically infected patients, revealing ASP as a HIV-1 antigen.

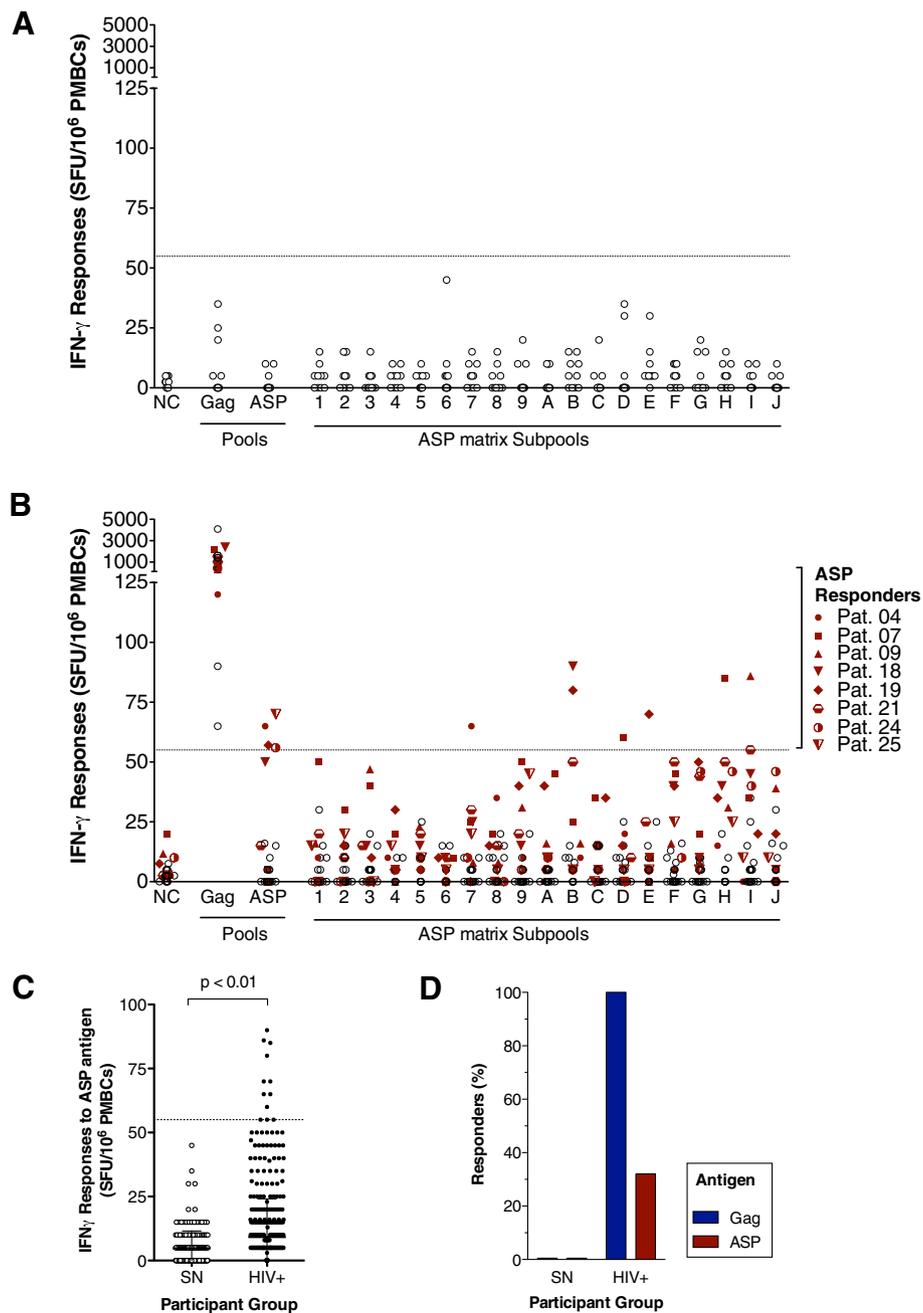
## Results

### ASP-overlapping peptides induce IFN- $\gamma$ T cell responses in HIV seropositive patients

Immune responses to ASP were first studied using PBMCs from HIV-1 subtype B seropositive patients (Pats.1 to 25, Additional file 1: Table S1) who were off antiretroviral therapy (ART) and 10 seronegative donors (SN). T cell responses were measured with an *ex vivo* interferon gamma (IFN- $\gamma$ )-ELISpot assay using a pool of peptides encompassing the whole ASP sequence (85 peptides, 14-18-mers overlapping by 10). To increase the likelihood of detecting CTL responses primed during acute or chronic infection, the ASP pool included peptides derived from the sequence of a transmitter founder virus WITO\_TF1 [39] and NL4-3, a viral strain isolated during chronic infection and expressing ASP in infected cells [29,33,37,38]. Within this cohort, 4 out of the 25 patients responded to ASP (Figure 1B, Pats.04, 19, 24, 25). A pool of peptides encompassing Gag was used as a positive control and induced CTL responses in all patients. No ASP-specific or Gag-specific responses were detected in any seronegative donors (Figure 1A). We then analyzed T cell responses to 19 subpools of ASP, containing 8 to 10 peptides (Additional file 2: Figure S1). We observed that 6 patients (Figure 1B, Pats 04, 07, 09, 18, 19, 21) responded to one or two subpools of ASP peptides and that a total of 6 different subpools induced CTL responses (#7, B, D, E, H, I). Thereafter, two patients (Pats.04 and 19) responded to both the total pool of ASP peptides as well as one or more subpools (subpools #7 and B, E). Notably, Pats.24 and 25 responses to subpools (G, H, J, and 9, respectively) tended to be positive though just under our stringent limit of positivity (more than four times background but 45 SFU/10<sup>6</sup> PBMCs). Overall, the magnitude of T cell responses to the ASP pool or subpools was significantly greater in seropositive patients than in seronegative donors ( $p < 0.01$ ; Figure 1C). Overall, we found a high frequency of patients responding to ASP antigens (32%; Figure 1D).

### Identification of ASP-specific HLA-A\*02 and -B\*07-restricted T cell responses

Our panels of OLP showed that HIV-1-specific IFN- $\gamma$  T cell responses were induced by ASP-derived antigens.



**Figure 1** HIV-1 ASP peptide pools activate IFN- $\gamma$  T cell responses in a cohort of HIV+ individuals. The magnitude of IFN- $\gamma$  ELISpot responses (SFU/10<sup>6</sup> PMBCs) to the Gag or ASP pools and subpools (1 to 9, and A to J) are shown for (A) seronegative donors and (B) HIV-1+ patients. Each dot represents one donor and mean IFN- $\gamma$  responses of triplicate or quadruplicate are indicated. The dashed line marks the standardized threshold (55 SFU/10<sup>6</sup> PMBCs) for positivity. (C) PBMCs were assessed for IFN- $\gamma$  production using ASP pools and 19 ASP subpools. Mann-Whitney U tests were performed to determine significant differences between the median responses to peptide pools ( $p < 0.01$ ). (D) The frequency of responders is illustrated as a proportion of seronegative (SN) or seropositive (HIV+) individuals with positive responses to the Gag pool (blue column) and either to the ASP pool or any ASP subpool (red column).

Attempts to further map ASP subpool responses failed to consistently identify individual epitopes or peptides that could contain them. However, the analysis was performed using 14-18-mer peptides for global sequence coverage of the ASP protein and may have provided suboptimal

targets for CD8+ T cells, which recognize MHC-I molecules presenting 8-11-mer peptides. We therefore investigated ASP-specific T cell responses using a common “reverse immunology” approach [17]. In brief, potential ASP-derived peptides with high affinity to a specific

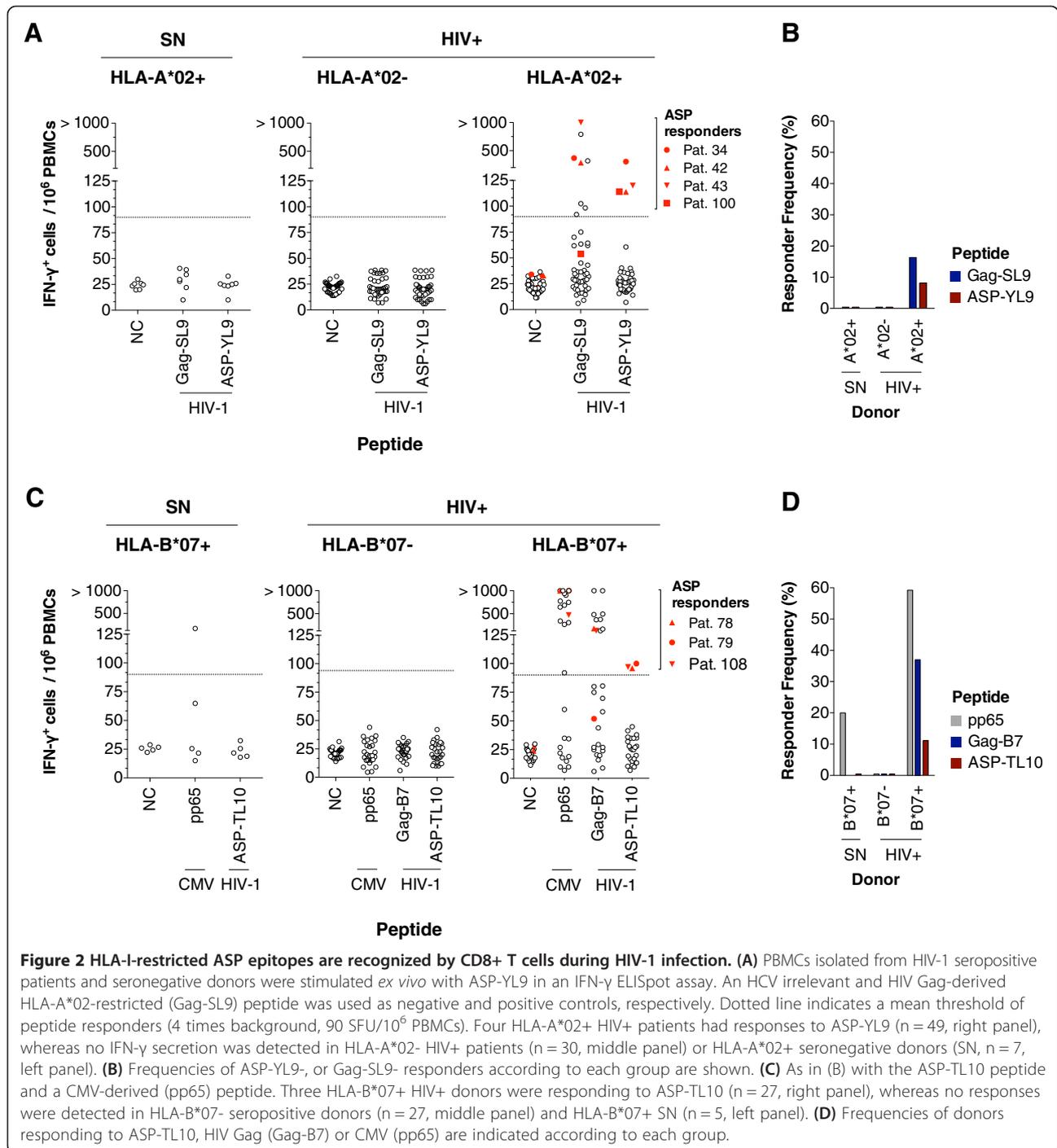
HLA-I allele were predicted, and T cell recognition of these candidates was evaluated in HLA-I-matched individuals. The analysis of HIV-1 clade B and M sequences revealed two peptide sequences, ASP-YL9 (YLYNSLLQL) and ASP-TL10 (TPNGSIFTTL), with high binding scores to HLA-A\*02 and HLA-B\*07 molecules, respectively (Additional file 3: Table S2). In comparing the conservation of classical epitopes and ASP peptides, both ASP peptides had classical equivalents that were either poorly (*e.g.* ASP-TL-10 and Gag-YF10) or highly (*e.g.* ASP-YL9 and Env-KL9) conserved [40-42]. To increase the probability of detecting HLA-I-restricted CTL responses to ASP, the ASP-YL9 and ASP-TL10 peptides were selected for use in immunoassays based on their high HLA-binding score and relative conservation. T cell responses against these peptides were investigated within a cohort of HIV-1 seropositive donors (Pats.32 to 126, Additional file 1: Table S1). PBMCs were screened for HLA-A\*02 and HLA-B\*07 alleles expression by antibody staining. PBMCs from 49 HLA-A\*02+ patients (Pats.32 to 74, 98 to 103) were assayed for *ex vivo* IFN- $\gamma$  ELISpot responses against the ASP-YL9 peptide. A known HIV-derived HLA-A\*02-restricted epitope (Gag-SL9) and an HCV-derived irrelevant peptide were used as positive and negative controls (NC), respectively (Figure 2A). Four patients (Pats.34, 42, and 43 and 100) responded to ASP-YL9. Magnitudes of ASP-specific T cell responses ranged from 114 to 307 SFU/10<sup>6</sup> PBMCs and were weaker than Gag-SL9 responses (92 to more than 1000 SFU/10<sup>6</sup> PBMCs). Strikingly, no responses against ASP-YL9 were detected in HLA-A\*02+ HIV-1 seronegative donors and in HLA-A\*02 negative patients (*n* = 30, Pats.89 to 95, 104 to 126). Height percent of PBMCs from HLA-A\*02+ seropositive donors were activated by ASP-YL9 (Figure 2B). We then assessed *ex vivo* IFN- $\gamma$  responses against the ASP-TL10 peptide. We used a pool of three known HLA-B\*07-restricted Gag epitopes (pool named as "Gag-B7") and a CMV-derived HLA-B\*07-restricted epitope (pp65) as positive controls. Twenty-seven HLA-B\*07+ patients were tested (Pats.69 to 88, 104 to 110) and three individuals (Pats.78, 79 and 108) positively responded to the ASP-TL10 antigen (average magnitude of responses of 98 SFU/10<sup>6</sup> PBMCs) (Figure 2C). No ASP-TL10-specific T cell responses were found within the HLA-B\*07+ seronegative donors and HLA-B\*07 negative HIV patients (*n* = 27, Pats.66 to 68; 96 to 103, 111 to 126). Eleven percent of PBMCs from HLA-B\*07+ seropositive donors were activated by ASP-TL10 (Figure 2D). Taken together, the frequency of responders to ASP-YL9 or ASP-TL10 was lower than the proportion of patients who responded to immunodominant Gag-derived antigens: 16% of HLA-A\*02+ patients responded to Gag-SL9 and 37% of HLA-B\*07+ patients responded to Gag-B7.

### ASP-specific CD8+ T cells produce multiple cytokines and chemokines

Recognition of 8-11-mer peptides by T cell receptors of CD8+ T cells requires presentation of these antigens by HLA-I molecules. To assess whether CD8+ T cells recognize the ASP-Y9L peptide, we analyzed production of intracellular cytokines in a flow cytometry assay (ICS) following stimulation of PBMCs from 11 HLA-A\*02+ patients (Pats.32 to 42), and one HLA-A\*02- patient as control (Pat.95). Cells were pulsed with the ASP-YL9 peptide. Production of MIP1- $\beta$  or IL-2, TNF $\alpha$  and IFN- $\gamma$  together was assessed on CD8+ cells (Figure 3A). We measured the proportion of activated cells producing at least one of these cytokines/chemokines upon peptide stimulation (Figure 3B). 0.1% of CD8+ T were specifically stimulated by ASP-YL9 for two HLA-A\*02+ patients (Pats.34 and 42). No ASP-specific stimulation of CD8+ T cells was observed for Pat.95 (HLA-A\*02-). Induction of Gag-SL9-specific CD8+ T cells was also detected in three patients, including Pats.34 and 42 (range, 0.1 to 0.3% of CD8+ T cells). The results strongly suggest that the IFN- $\gamma$  detected in our previous ELISpot assays was secreted by CD8+ T cells (Figure 2A, B). We then evaluated whether ASP-specific CD8+ T cells were producing multiple cytokines together (Figure 3C). 97% of ASP-YL9-specific CD8+ T cells were producing IL-2, IFN- $\gamma$  or TNF $\alpha$ , together with a production of MIP1- $\beta$  for 80% of them (Figure 3D, black pie). Thus, epitope-specific CD8+ T cells produce multiple cytokine/chemokines upon recognition of ASP-YL9. This subset exhibits a functional profile similar to Gag-SL9-specific CD8+ T cells, with a majority of cells producing multiple cytokine/chemokines at the same time (Figure 3D). Although the measurable cytokines are not evidence of T cell-mediated cytotoxicity, the capacity of ASP-specific CD8+ T cells to produce multiple cytokines/chemokines possibly enhances their ability to kill HIV-1-infected cells [43,44].

### Identification of ASP-YL9-specific CD8+ T cells by tetramer staining

We further examined the antigen specificity of CD8+ T cell responses in a subset of participants (Pats.26 to 31). We previously showed that magnitude of HIV-1 ARF-specific CD8+ T cell responses are increased following *in vitro* stimulation of PBMCs with antigens [45]. PBMCs from HLA-A\*02+ HIV+ patients and seronegative control donors (SN) were expanded with the ASP-YL9 peptide and stained with an ASP-YL9/HLA-A\*02 tetramer. When PBMCs from Pat.28 were analyzed, 0.11% of CD8+ T cells were stained specifically with the HLA-A\*02/ASP-YL9 tetramer, corresponding to 0.06% of the total T cell population (Figure 4A, B). In contrast, no specific staining could be detected within the CD4+ T cell population (Figure 4A). No tetramer staining was detected in other



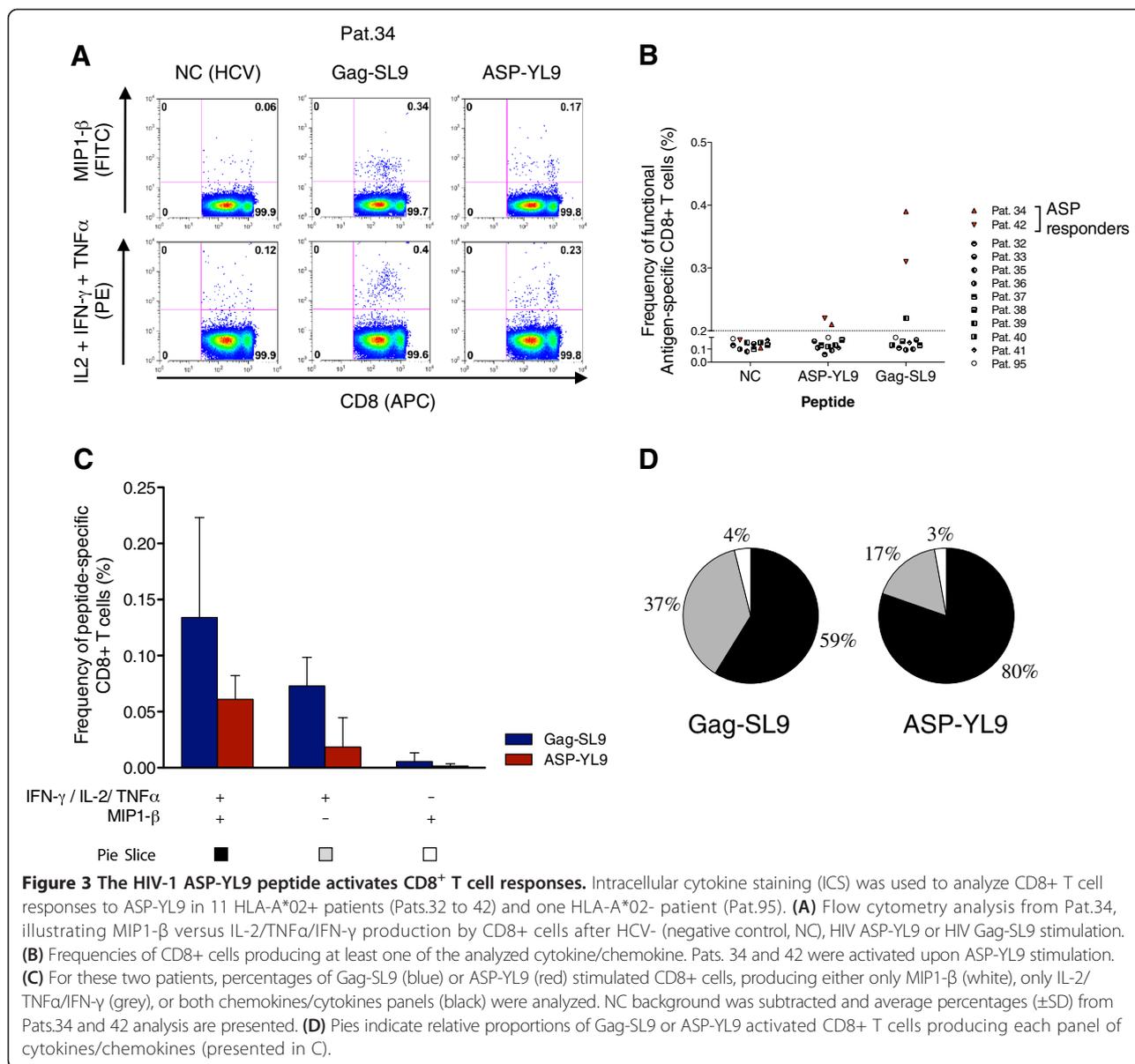
patients and seronegative donors. Together, these results indicate that the ASP-YL9 peptide elicits HLA-A\*02 restricted CD8+ T cells during HIV infection.

**Discussion**

The existence of a tenth HIV-1 protein encoded by genomic plus strand has been suggested since the late 80s [27]. Yet, *in vivo* antisense transcription and translation of not only HIV-1, but also of other retroviruses, has

remained a topic of debate. In this study, we show that CD8 T cell responses to an antisense derived HIV protein (ASP) are detected during chronic HIV infection. Further, we demonstrate that these ASP specific CD8 T cells have comparable functionality to the traditional protein-derived HLA-I restricted epitopes.

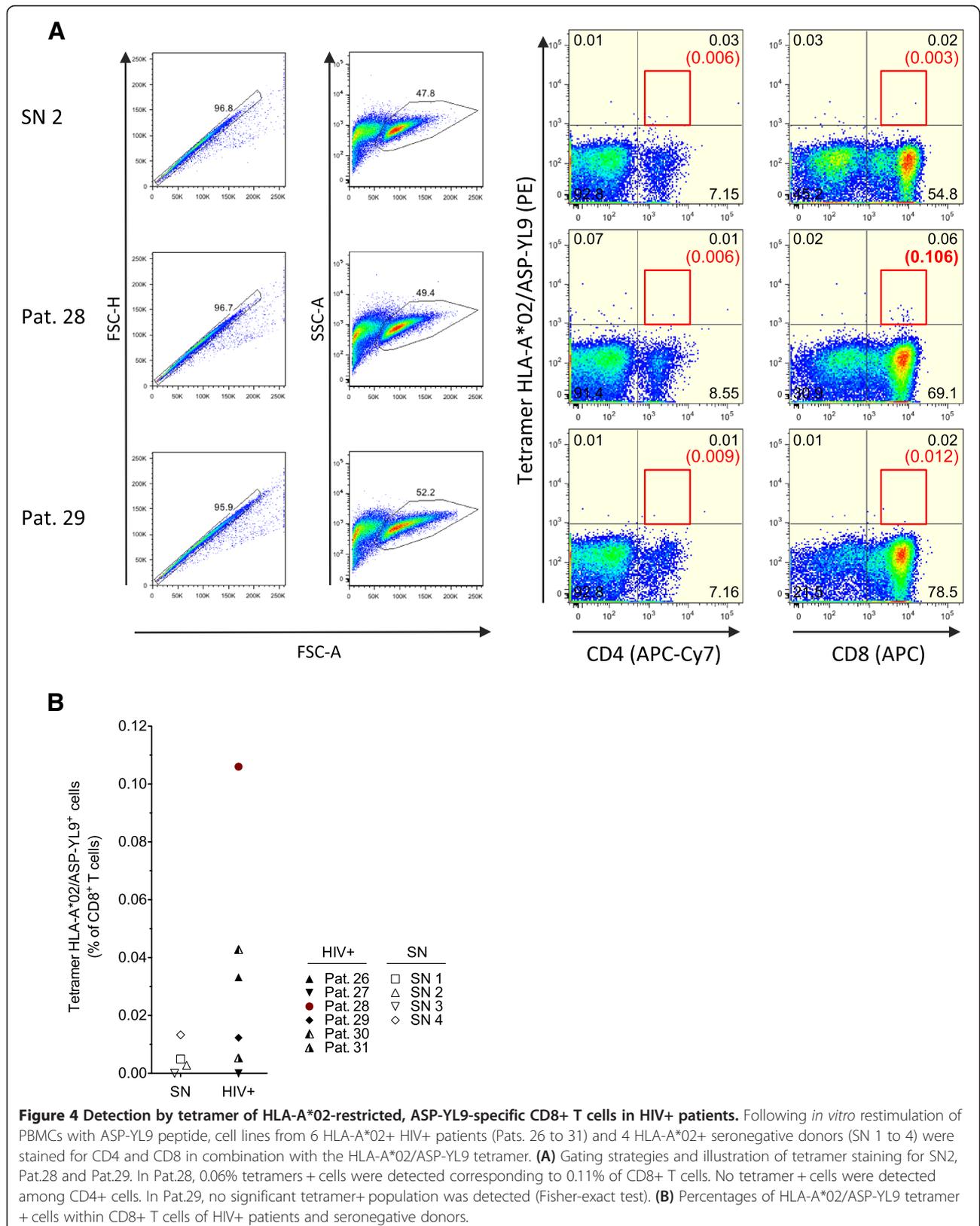
Virological but also immunological attributes of antisense proteins were emphasized by HBZ and APH-2, two antisense strand-encoded proteins from the human



T cell leukemia virus type 1 (HTLV-1) and HTLV-2, respectively [46-48]. HBZ modulates viral transcription, persistence of the virus and infectivity. It may also play a role in induction of cellular immune responses controlling the virus [49]. Thus, antisense proteins appear critical for understanding retroviral pathogenesis. The role of antisense transcripts from HIV-1 still remains poorly studied. Their existence was demonstrated *in vitro* and in the context of infection [32,50]. Conservation of the antisense reading frame directed investigations to search for a protein translated by these antisense transcripts [27,35,38]. Vaquero *et al.* demonstrated that antibodies from sera of seropositive individuals were detecting ASP [33]. Evidence of the expression of ASP was further confirmed by *in vitro* experiments [29,34,37]. Although all

these approaches stressed the fact that ASP is expressed during infection, studies have not convinced the scientific community of the existence of this protein. In the present study, we revisited this question by investigating the induction of ASP-specific CD8<sup>+</sup> T cells in patients after HIV-1 infection. Our results show for the first time that a proportion of HIV-specific CD8<sup>+</sup> T cells are targeting ASP and are activated during chronic infection.

Over the last three decades, the pool of HIV-1 CTL epitopes has been studied extensively. CTLs target epitopes from all nine structural and regulatory proteins encoded by open reading frames in the RNA sense strand. Alternative translation of retroviral genes was initially hypothesized in mice infected by LMP5, an immunodeficiency-causing retrovirus [51]. Green and



colleagues expanded the known pool of T cell targets by demonstrating that CTL epitopes were generated *in vivo* from translation of a *gag* ARF, rather than the gene's primary reading frame. ARF-derived antigens, often smaller in size than traditional proteins, appear labile and difficult to detect by conventional approaches. Nevertheless, the incredible sensitivity and specificity of CTLs allows for efficient detection of cryptic epitopes (CE) from ARFs. CTLs targeting CE of HIV-1 and SIV have been detected by several groups, thus revealing the translation of sense and antisense ARFs [15-17,52,53]. CTLs targeting CE are elicited during infection following stimulation by antigen-presenting cells. Further, mutations within these CE can arise in order to escape CTL responses [11,53]. All of these findings strongly suggest that ARF sequences are translated during the viral cycle and subsequently recognized by the immune system. Here, we demonstrated that ASP-specific CD8<sup>+</sup> T cells were detectable in the blood of seropositive individuals by combining two complementary approaches. Strikingly, no epitope-specific T cell responses were detected in seronegative donors or in HIV<sup>+</sup> patients who did not carry the appropriate HLA-I alleles. Peptides from various locations within ASP were recognized. Our results indicate that the full-length ASP protein is likely processed and presented by MHC-I molecules. Since ASP-specific CD8<sup>+</sup> T cells are generated in infected individuals during the course of HIV-1 infection, our results strongly suggest that ASP is expressed by infected cells and constitutes a source of antigens for inducing CD8<sup>+</sup> T cell responses, either following natural infection or vaccination.

In our unbiased approach, approximately 30% of the donors showed positive IFN- $\gamma$  responses to ASP, whereas the response level to individual peptides ASP-YL9 and ASP-TL10 were detected in 8% of HLA-A\*02<sup>+</sup> donors and 11% of HLA-B\*07<sup>+</sup> donors, respectively. The proportion of responses to pool and subpools may have been higher than the proportion of responses to optimal peptides because pools include a greater number of peptides, thus increasing the likelihood of activating ASP-specific CTLs with various antigenic specificities. Our lead hypothesis was that ASP is expressed during infection and induces CD8<sup>+</sup> T cell responses. However, whether ASP is expressed during acute and/or chronic infections remains to be determined. Thereafter, we thought that including peptides overlapping WITO\_TF1 sequence might increase the chance to activate CTL responses primed during the early step of acute infection. In addition, NL4-3, that was used to design the set of peptides overlapping a viral strain isolated during chronic infection, was selected because we have previously shown that NL4-3-infected cells express ASP [29,37,38]. WITO\_TF1 and NL4-3 might not be representative of the circulating virus present in the tested patients but allowed us to maximize our chance to

detect CTL responses. Obviously, it would be of great interest to sequence the quasi-species circulating in the blood of patients to study the conservation of ASP ORF and amino acid sequences with regards to ASP-specific CTL responses.

As majority of our patients were treated, the frequency of ASP responders was doubtlessly underestimated. ART may impact the magnitude and the breadth of ASP-specific CTL responses, as demonstrated for ARF sense and antisense epitopes [54]. The magnitude of T cell responses to ASP appear weak but were significantly higher than non-specific responses from seronegative donors. Although responses to the ASP peptide pool and subpools did not exceed to 100 SFU/10<sup>6</sup> PBMCs, ASP-YL9 responses reached 300 SFU/10<sup>6</sup> of PBMCs. The capacity of peptide pools to activate T cell responses was evaluated using cryopreserved PBMCs, whereas fresh PBMCs were used to detect ASP-YL9 and ASP-TL10 responses. Although the magnitudes of CTL responses might have been stronger using fresh samples, using frozen cells, we readily detected ASP-specific CTL responses. Magnitudes of ASP-specific responses from *ex vivo* PBMCs are in fact consistent with previous analyses for responses to cryptic epitopes from ARFs [15-17,45]. However, the limited magnitude of T cell responses may be due to low expression of these antigens, poor MHC-I binding and presentation, or a T cell repertoire with a low affinity for these antigens. Indeed, in contrast to Gag, cryptic epitopes are produced by non-conventional mechanisms of translation of the viral genome. To our knowledge, events responsible for protein synthesis from ARF of transcripts and mechanisms regulating this process are not clearly defined. The transcription factors NF $\kappa$ B and Sp1 but not the HIV-1 Tat protein are positive modulators of HIV antisense transcription [55]. Antisense RNA appeared preferentially transcribed in specific cell-types (e.g. monocyte-derived cells) [37]. ASP potentially induces autophagy [38]. Thus, various parameters may justify presumed low abundance of ASP in infected cells, and therefore a weak induction of CTL responses. HIV-specific CTLs conferring protection to patients have been shown to have a high sensitivity to antigen, develop multiple antiviral functions and have non-exhausted phenotype [56]. In our approach, we showed by intracellular staining that production of cytokine/chemokines by ASP-specific CTLs appears as diverse as for conventional CTLs (Gag-SL9). The quality of ASP-specific CTLs responses, their phenotypes and capacity to control infection remains to be defined.

We focused our explorations on individuals expressing HLA-A\*02 or HLA-B\*07, two alleles frequent among the Caucasian population and whose peptide anchor motifs are well defined. These alleles are not associated with a delayed disease progression. It would be of interest to analyze ASP-specific CTL responses in patients

that spontaneously control the infection but also in post-treatment controllers [57]. Current vaccines do not prioritize the expression of ASP or epitopes from this ARF. Harnessing the strategic engineering and control of HIV-1's genetic code in the context of a highly efficient vector has the potential to increase production and presentation of viral immunogens following vaccination. Importantly, the number of vector-derived immunogens could be increased through constructs that express both sense and antisense reading frames, thus inducing broader immune responses to protein- and ARF-derived epitopes without expanding the size of the vector insert [5,58-63]. In fact, vector-derived cryptic epitopes encoded by antisense *gag* and *pol* ARFs have been shown to induce HIV-1-specific T cell responses in vaccinees who participated in a Phase 2b trial (HVTN 205). This suggests that current MVA/DNA vaccines encoding the *env* region may already induce ASP-specific CD8+ T cell responses [45].

## Conclusions

Our data demonstrated for the first time that epitope-specific CD8+ T cells from infected individuals target ASP. Moreover, the *ex vivo* induction of ASP-specific CD8+ T cell responses provides strong evidence of ASP expression during infection, thereby supporting ARFs as an additional source of HIV-1 immunogens. Future studies assessing the functionality of ASP and other conserved ARF sequences will therefore be critical for enhancing our understanding of viral life cycle and developing effective HIV-1 vaccines.

## Methods

### Study populations

Two cohorts of HIV infected patients were studied. First, peripheral blood mononuclear cells (PBMCs) were collected from US patients with HIV-1 clade B infections (Additional file 1: Table S1, Pat 1 to 25) and seronegative donors ( $n = 10$ ). All HIV seropositive patients were off ART and, with a median CD4 T cell count of 789 cells/ $\mu$ L (range, 449 – 1176 cells/ $\mu$ L), median CD4 nadir of 434 cells/ $\mu$ L (range, 116 – 827 cells/ $\mu$ L), and median plasma viral load (VL) of 616 RNA copies/mL (range, 21 – 16,400 RNA copies/mL). HLA class I alleles were typed to two- or four-digit specificity by PCR amplification using sequence-specific primers as described before [64].

Second, PBMCs were collected from HIV-1 positive HCV (Hepatitis C virus) negative patients from a French ANRS cohort (Additional file 1: Table S1, Pats. 26 to 126) [65]. Ninety-two percent of these patients were on ART (range, 0.5 - 26 years). Twenty-five percent of all patients had a detectable viral load (range, 21–2,951,507 RNA copies/mL) and the median CD4 T cell count was 522 (range, 18 – 1854 cells/ $\mu$ L). All patients were chronically infected and 8 were recently detected as seropositive

(Pats. 26, 42, 50, 70, 87, 100, 107, 126). HLA types were first determined by FACS analysis using anti-HLA\*07 (BB7.1, AbD Serotec) and anti-HLA-A\*02 (BB7.2, BioLegend) antibodies. HLA-B\*07+ and HLA-A\*02+ cell lines were simultaneously analyzed as positive controls. HLA status of ASP responders was further confirmed by genotyping using PCR or using the Luminex xMAP technology [66,67]. PBMCs from HIV-1-seronegative control donors were purchased from the Etablissement Français du Sang of the Pitié-Salpêtrière Hospital (Paris, France).

Demographic, HLA-typing, virological and clinical characteristics of patients included in both cohorts are shown in Additional file 1: Table S1.

### Ethics statement

All samples were obtained according to protocols approved by the Institutional Review Board (IRB) at the University of Alabama at Birmingham. Patient samples were collected according to French Ethical rules. Written informed consent and approval by institutional review Board at the Pitié-Salpêtrière Hospital were obtained.

### Antigen design

Consensus clade B 15-mer peptides (123 total) overlapping by 11 amino acids (aa) for HIV-1 Gag were obtained through the NIH AIDS Reagent Program (catalog #8117). In our unbiased approach, we used ASP sequences obtained from the transmitted founder virus of an acute patient (WITO\_TF1, Genbank Acc.# JN944938.1) and a HIV-1 lab strain, NL4.3 (Genbank Acc.# M19921), to design 85 overlapping ASP peptides (OLPs; 14 to 18mers overlapping by 10 residues) using the Los Alamos National Lab's PeptGen tool (<http://www.hiv.lanl.gov/content/sequence/PEPTGEN/peptgen.html>; Additional file 2: Figure S1). Peptides were synthesized in a 96 well array format by New England Peptide [68]. Individual OLPs were combined into a single Gag or ASP pool. ASP subpools of no more than 10 peptides were used in a matrix format. Peptides were reconstituted in 100% DMSO at 40 mM/peptide. Working stocks were prepared with water to a final concentration of 100  $\mu$ M/peptide. The final concentration during the ELISpot assay was 5  $\mu$ M/peptide in 100  $\mu$ l, corresponding to  $\leq 1\%$  and  $\leq 0.1\%$  of DMSO (v/v) for the total pools (ASP and Gag) and ASP subpools, respectively.

As a complementary strategy, HLA-I binding affinities and associations of potential ASP epitopes were evaluated using BIMAS, SYFPEITHI and NetMHC prediction algorithms (Additional file 3: Table S2) [69-71]. Two epitopes were synthesized, YLYNSLLQL (ASP-YL9) and TPNGSIFTTL (ASP-TL10) (Proimmune). Peptides were selected based on high binding affinities for HLA-I alleles HLA-A\*02 and HLA-B\*07 respectively, and on the other hand on their conservation among HIV-1 strains.

All peptides used in this study were >85% purity, as shown by HPLC profiles.

#### **Ex vivo IFN- $\gamma$ ELISpot assay**

In the unbiased approach using OLP, cryopreserved PBMCs were thawed and rested overnight at 37°C, 5% CO<sub>2</sub> in RPMI containing 5% human serum and supplemented with penicillin, streptomycin, and L-glutamine. Interferon gamma ELISpot assays were performed as previously described using a total pool containing all peptides, subpools, or individual peptides [15,45]. Antigens were tested in duplicate or triplicate with 100,000 PBMCs/well in nitrocellulose 96-well plates precoated overnight with anti-IFN- $\gamma$  coating antibody and unstimulated cells were plated in quadruplicate as a negative control; phytohemagglutinin (PHA) was tested in duplicate as a positive control. Spot forming units (SFU) were counted by an ELISpot reader (ImmunoSpot; CTL) as a measure of cytokine production.

In the approach using optimal peptides, *ex vivo* ficoll-isolated PBMCs were stimulated by 1  $\mu$ M synthetic peptides in IFN- $\gamma$  ELISpot assays as previously described [72]. An HCV-derived epitope (GPRLGVRAT) was used as negative control (NC), and a CMV-derived epitope (pp65<sub>417</sub>TPRVTGGGAM<sub>426</sub>) as positive control for HLA-B\*07 donors. Known HIV-1 Gag-derived epitopes were used to test HIV reactivity, either a pool (Gag-B7) of three HLA-B\*07-restricted epitopes (Gag-SV9, p24<sub>16</sub>SPRTLNAWV<sub>24</sub>, Gag-TL9, p24<sub>48</sub>TPQDLNTML<sub>56</sub>, Gag-YF9, p2p7p1p6<sub>121</sub>YPLASLRS<sub>130</sub>) or an HLA-A\*02-restricted epitope (Gag-SL9, p17<sub>77</sub>SLYNTVATL<sub>85</sub>) [41,73].

T cell responses were reported as positive if i) the mean response to the sample's negative control was inferior to 30 SFU/10<sup>6</sup> PMBCs, ii) the average response was more than 55 SFU/10<sup>6</sup> PMBCs, iii) greater than 4 times the mean response to the sample's negative control, iv) greater than the mean response to the sample's negative control plus 3 standard deviations, and v) the mean response to the sample's positive control (PHA) was greater than 500 SFU/10<sup>6</sup> PMBCs [74].

#### **Intracellular cytokine staining (ICS) assay**

*Ex vivo* isolated PBMCs from 11 HLA-A\*02+ and 1 HLA-A\*02- HIV donors were rested overnight in RPMI 1640 supplemented with 5% human serum (Institut Jacques Boy), 2 mM L-glutamine, 100 IU/mL penicillin, 100  $\mu$ g/mL streptomycin, 10 mM HEPES, 1% nonessential amino acids, 1 mM sodium pyruvate. Cells were pulsed with individual peptides (1  $\mu$ M) for 2 hours at 37°C and brefeldin A (5  $\mu$ g/mL; Molecular Probes) was added for 6 hours at 37 °C. Cells were fixed, permeabilized, and stained using standard procedures with CD8-APC (RPA-T8), TNF $\alpha$ -PE (MAb11), IL-2-PE (MQ1-17H12), IFN- $\gamma$ -PE (B27) (BD Pharmingen), and MIP-1 $\beta$ -FITC (24006, R&D

Systems). Cells were analyzed with a FACS-Calibur™ cytometer (BD Biosciences). Flow cytometry data were analyzed with FlowJo software (Tree Star).

#### **In vitro expansion of ASP-specific CD8+ T cells**

Cryopreserved PBMCs from 6 HIV-1+ patients and 4 seronegative donors with HLA-A\*02 alleles were pulsed with the ASP-YL9 peptide (1  $\mu$ M) and cultured for 7 days in the presence of IL-2 (100 IU/mL; Miltenyi). On day 3, 20 ng/ml recombinant human IL-7 (Sanofi-Synthelabo) was added. Samples were then evaluated for antigen specificity in a flow cytometry-based assay using anti-CD4-APC-Cy7 (RPA-T4, BD Pharmingen), anti-CD8-APC (RPA-T8 BD Pharmingen), and PE-conjugated HLA-A\*02 tetramers presenting the ASP-YL9 peptide (TC Metrix). Cultured cells were incubated in PBS 0.5% BSA (Sigma) with tetramers and antibodies for 20 min at 4°C, then washed and fixed before measuring the frequency of single CD4- tetramer + CD8+ T cells for each participant with a BD FACS Canto cytometer.

#### **Statistical analysis**

T cell responses were analyzed for differences in magnitude using two-tailed non-parametric Mann–Whitney *U* tests calculated by GraphPad Prism v6.0b and frequency comparisons were done using two-tailed Fisher's exact tests calculated by GraphPad QuickCalcs, where *p* values of 0.00001 or less were considered statistically significant. A high-powered statistical test and an adjusted significance threshold were applied to stringently assess small differences between large sample sizes (total number of subset and gated events) and Type I errors.

#### **Additional files**

**Additional file 1: Table S1.** Demographic, HLA-typing, virological and clinical characteristics of patients.

**Additional file 2: Figure S1.** Antigens designed for detection of epitope-specific T cell responses provide global sequence coverage of HIV-1 ASP. (A) Overlapping peptides were designed using ASP sequences isolated from transmitted founder virus of an acute patient (WITO\_TF1, red) and a HIV-1 lab strain (NL4-3, green). Peptides encoded by both strains are indicated in purple. Amino acid differences between peptides are highlighted in bold. Translated sequences are shown in the 5' to 3' direction, corresponding to the N- and C-termini of ASP, respectively. Sequences for ASP-YL9 and ASP-TL10 peptides are boxed. ni: not included, if the peptide could not be synthesized or did not pass NEP's quality control standards. (B) Matrix design for subpools ASP peptides (A to J, and 1 to 9). Peptide number refers to the peptides listed in (A). Patients responding to peptide subpools are indicated along the x- and right y-axis.

**Additional file 3: Table S2.** Binding affinity and conservation of HLA-A\*02- and HLA-B\*07-restricted HIV-1 epitopes.

#### **Abbreviations**

HIV-1: Human immunodeficiency virus type 1; ASP: HIV-1 antisense protein; HLA-I: Human leukocyte antigen class I; MHC-I: Major histocompatibility class

I molecule; ARF: Alternative reading frame; ORF: Open reading frame; OLP: Overlapping peptides; CE: Cryptic epitope.

### Competing interests

The authors declare that they have no competing interests.

### Authors' contributions

A Bet performed research, analyzed data, and drafted the manuscript. A Bansal guided the experimental design, reviewed the data analysis, and revised the manuscript. A Bansal and EAM equally contributed to this work. SS collected data, performed research, and analyzed data. AG and JMM analyzed viral sequences. IT performed HLA genotyping. AS, AG and CK enrolled patients and collected clinical data. EAM, SGD and SC performed experiments. A. Bansal, AM PAG and SC pioneered the collaboration between research teams. They conceived the study, directed research aims, and revised the manuscript. All authors read and approved the final manuscript.

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### References

- Schmitz JE, Kuroda MJ, Santra S, Sasseville VG, Simon MA, Lifton MA, et al. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science*. 1999;283:857–60.
- Jin X, Bauer DE, Tuttleton SE, Lewin S, Gettie A, Blanchard J, et al. Dramatic rise in plasma viremia after CD8 (+) T cell depletion in simian immunodeficiency virus-infected macaques. *J Exp Med*. 1999;189:991–8.
- Borrow P, Lewicki H, Hahn BH, Shaw GM, Oldstone MB. Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J Virol*. 1994;68:6103–10.
- Wang YE, Li B, Carlson JM, Streeck H, Gladden AD, Goodman R, et al. Protective HLA class I alleles that restrict acute-phase CD8+ T-cell responses are associated with viral escape mutations located in highly conserved regions of human immunodeficiency virus type 1. *J Virol*. 2009;83:1845–55.
- Wilson NA, Keele BF, Reed JS, Piaskowski SM, MacNair CE, Bett AJ, et al. Vaccine-induced cellular responses control simian immunodeficiency virus replication after heterologous challenge. *J Virol*. 2009;83:6508–21.
- Migueles SA, Sabbaghian MS, Shupert WL, Bettinotti MP, Marincola FM, Martino L, et al. HLA B\*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. *Proc Natl Acad Sci U S A*. 2000;97:2709–14.
- Altfield M, Addo MM, Rosenberg ES, Hecht FM, Lee PK, Vogel M, et al. Influence of HLA-B57 on clinical presentation and viral control during acute HIV-1 infection. *AIDS*. 2003;17:2581–91.
- Carlson JM, Brumme ZL, Rousseau CM, Brumme CJ, Matthews P, Kadie C, et al. Phylogenetic dependency networks: inferring patterns of CTL escape and codon covariation in HIV-1 Gag. *PLoS Comput Biol*. 2008;4:e1000225.
- Goepfert PA, Lum W, Farmer P, Matthews P, Prendergast A, Carlson JM, et al. Transmission of HIV-1 Gag immune escape mutations is associated with reduced viral load in linked recipients. *J Exp Med*. 2008;205:1009–17.
- Kloverpris HN, Stryhn A, Harndahl M, Carlson JM, Leslie AJ, Chen F, et al. HLA-A\*68:02-restricted Gag-specific cytotoxic T lymphocyte responses can drive selection pressure on HIV but are subdominant and ineffective. *AIDS*. 2013;27:1717–23.
- Cardinaud S, Consiglieri G, Bouziat R, Urrutia A, Graff-Dubois S, Fourati S, et al. CTL escape mediated by proteasomal destruction of an HIV-1 cryptic epitope. *Plos Pathogens*. 2011;7:e1002049.
- Goulder PJ, Brander C, Tang Y, Tremblay C, Colbert RA, Addo MM, et al. Evolution and transmission of stable CTL escape mutations in HIV infection. *Nature*. 2001;412:334–8.
- Perreau M, Levy Y, Pantaleo G. Immune response to HIV. *Curr Opin HIV AIDS*. 2013;8:333–40.
- Bullock TN, Eisenlohr LC. Ribosomal scanning past the primary initiation codon as a mechanism for expression of CTL epitopes encoded in alternative reading frames. *J Exp Med*. 1996;184:1319–29.
- Bansal A, Carlson J, Yan J, Akinsiku OT, Schaefer M, Sabbaj S, et al. CD8 T cell response and evolutionary pressure to HIV-1 cryptic epitopes derived from antisense transcription. *J Exp Med*. 2010;207:51–9.
- Berger CT, Carlson JM, Brumme CJ, Hartman KL, Brumme ZL, Henry LM, et al. Viral adaptation to immune selection pressure by HLA class I-restricted CTL responses targeting epitopes in HIV frameshift sequences. *J Exp Med*. 2010;207:61–75.
- Cardinaud S, Moris A, Fevrier M, Rohrlisch PS, Weiss L, Langlade-Demoyen P, et al. Identification of cryptic MHC I-restricted epitopes encoded by HIV-1 alternative reading frames. *J Exp Med*. 2004;199:1053–63.
- Godet Y, Moreau-Aubry A, Guilloux Y, Vignard V, Khammari A, Dreno B, et al. MELOE-1 is a new antigen overexpressed in melanomas and involved in adoptive T cell transfer efficiency. *J Exp Med*. 2008;205:2673–82.
- Hilburn S, Rowan A, Demontis MA, MacNamara A, Asquith B, Bangham CR, et al. In vivo expression of human T-lymphotropic virus type 1 basic leucine-zipper protein generates specific CD8+ and CD4+ T-lymphocyte responses that correlate with clinical outcome. *J Infect Dis*. 2011;203:529–36.
- Chen W, Calvo PA, Malide D, Gibbs J, Schubert U, Bacik I, et al. A novel influenza A virus mitochondrial protein that induces cell death. *Nat Med*. 2001;7:1306–12.
- Faggioni G, Ciannamaroni A, De Santis R, Pomponi A, Scicluna MT, Barbaro K, et al. Evidence of a humoral response to a novel protein WARF4 embedded in the West Nile virus NS4B gene encoded by an alternative open reading frame. *Int J Mol Med*. 2009;23:509–12.
- Maness NJ, Walsh AD, Piaskowski SM, Furlott J, Kolar HL, Bean AT, et al. CD8+ T cell recognition of cryptic epitopes is a ubiquitous feature of AIDS virus infection. *J Virol*. 2010;84:11569–74.
- Mayrand SM, Healy PA, Torbett BE, Green WR. Anti-Gag cytolytic T lymphocytes specific for an alternative translational reading frame-derived epitope and resistance versus susceptibility to retrovirus-induced murine AIDS in F(1) mice. *Virology*. 2000;272:438–49.
- Saulquin X, Scotet E, Trautmann L, Peyrat MA, Halary F, Bonneville M, et al. +1 Frameshifting as a novel mechanism to generate a cryptic cytotoxic T lymphocyte epitope derived from human interleukin 10. *J Exp Med*. 2002;195:353–8.
- Wang RF, Parkhurst MR, Kawakami Y, Robbins PF, Rosenberg SA. Utilization of an alternative open reading frame of a normal gene in generating a novel human cancer antigen. *J Exp Med*. 1996;183:1131–40.
- Garrison KE, Champiat S, York VA, Agrawal AT, Kallas EG, Martin JN, et al. Transcriptional errors in human immunodeficiency virus type 1 generate targets for T-cell responses. *Clin Vaccine Immunol*. 2009;16:1369–71.
- Miller RH. Human immunodeficiency virus may encode a novel protein on the genomic DNA plus strand. *Science*. 1988;239:1420–2.
- Michael NL, Vahey MT, d'Arcy L, Ehrenberg PK, Mosca JD, Rappaport J, et al. Negative-strand RNA transcripts are produced in human immunodeficiency virus type 1-infected cells and patients by a novel promoter downregulated by Tat. *J Virol*. 1994;68:979–87.

29. Clerc I, Laverdure S, Torresilla C, Landry S, Borel S, Vargas A, et al. Polarized expression of the membrane ASP protein derived from HIV-1 antisense transcription in T cells. *Retrovirology*. 2011;8:74.
30. Lefebvre G, Desfarges S, Uyttebroeck F, Munoz M, Beerenwinkel N, Rougemont J, et al. Analysis of HIV-1 expression level and sense of transcription by high-throughput sequencing of the infected cell. *J Virol*. 2011;85:6205–11.
31. Schopman NC, Willemsen M, Liu YP, Bradley T, van Kampen A, Baas F, et al. Deep sequencing of virus-infected cells reveals HIV-encoded small RNAs. *Nucleic Acids Res*. 2012;40:414–27.
32. Kobayashi-Ishihara M, Yamagishi M, Hara T, Matsuda Y, Takahashi R, Miyake A, et al. HIV-1-encoded antisense RNA suppresses viral replication for a prolonged period. *Retrovirology*. 2012;9:38.
33. Vanhee-Brossollet C, Thoreau H, Serpente N, D'Auriol L, Levy JP, Vaquero C. A natural antisense RNA derived from the HIV-1 env gene encodes a protein which is recognized by circulating antibodies of HIV+ individuals. *Virology*. 1995;206:196–202.
34. Briquet S, Vaquero C. Immunolocalization studies of an antisense protein in HIV-1-infected cells and viral particles. *Virology*. 2002;292:177–84.
35. Barbagallo MS, Birch KE, Deacon NJ, Mosse JA. Potential control of human immunodeficiency virus type 1 asp expression by alternative splicing in the upstream untranslated region. *DNA Cell Biol*. 2012;31:1303–13.
36. Gao F, Li Y, Decker JM, Peyrel FW, Bibollet-Ruche F, Rodenburg CM, et al. Codon usage optimization of HIV type 1 subtype C gag, pol, env, and nef genes: in vitro expression and immune responses in DNA-vaccinated mice. *AIDS Res Hum Retroviruses*. 2003;19:817–23.
37. Laverdure S, Gross A, Arpin-Andre C, Clerc I, Beaumelle B, Barbeau B, et al. HIV-1 antisense transcription is preferentially activated in primary monocyte-derived cells. *J Virol*. 2012;86:13785–9.
38. Torresilla C, Larocque E, Landry S, Halin M, Coulombe Y, Masson JY, et al. Detection of the HIV-1 minus-strand-encoded antisense protein and its association with autophagy. *J Virol*. 2013;87:5089–105.
39. Ochsenbauer C, Edmonds TG, Ding H, Keele BF, Decker J, Salazar MG, et al. Generation of transmitted/founder HIV-1 infectious molecular clones and characterization of their replication capacity in CD4 T lymphocytes and monocyte-derived macrophages. *J Virol*. 2012;86:2715–28.
40. Firat H, Tourdot S, Ureta-Vidal A, Scardino A, Suhrbier A, Buseyne F, et al. Design of a polyepitope construct for the induction of HLA-A0201-restricted HIV 1-specific CTL responses using HLA-A\*0201 transgenic, H-2 class I KO mice. *Eur J Immunol*. 2001;31:3064–74.
41. Cardinaud S, Bouziat R, Rohrlisch PS, Tourdot S, Weiss L, Langlade-Demoyen P, et al. Design of a HIV-1-derived HLA-B07.02-restricted polyepitope construct. *AIDS*. 2009;23:1945–54.
42. Corbet S, Nielsen HV, Vinner L, Lauemoller S, Therrien D, Tang S, et al. Optimization and immune recognition of multiple novel conserved HLA-A2, human immunodeficiency virus type 1-specific CTL epitopes. *J Gen Virol*. 2003;84:2409–21.
43. Akinsiku OT, Bansal A, Sabbaj S, Heath SL, Goepfert PA. Interleukin-2 production by polyfunctional HIV-1-specific CD8 T cells is associated with enhanced viral suppression. *J Acquir Immune Defic Syndr*. 2011;58:132–40.
44. Betts MR, Nason MC, West SM, De Rosa SC, Migueles SA, Abraham J, et al. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. *Blood*. 2006;107:4781–9.
45. Bet A, Sterrett S, Sato A, Bansal A, Goepfert PA. Characterization of T-cell responses to cryptic epitopes in recipients of a noncodon-optimized HIV-1 vaccine. *J Acquir Immune Defic Syndr*. 2014;65:142–50.
46. Halin M, Douceron E, Clerc I, Journo C, Ko NL, Landry S, et al. Human T-cell leukemia virus type 2 produces a spliced antisense transcript encoding a protein that lacks a classic bZIP domain but still inhibits Tax2-mediated transcription. *Blood*. 2009;114:2427–38.
47. Gaudray G, Gachon F, Basbous J, Biard-Piechaczyk M, Devaux C, Mesnard JM. The complementary strand of the human T-cell leukemia virus type 1 RNA genome encodes a bZIP transcription factor that down-regulates viral transcription. *J Virol*. 2002;76:12813–22.
48. Arnold J, Yamamoto B, Li M, Phipps AJ, Younis I, Lairmore MD, et al. Enhancement of infectivity and persistence in vivo by HBZ, a natural antisense coded protein of HTLV-1. *Blood*. 2006;107:3976–82.
49. Macnamara A, Rowan A, Hillburn S, Kadolsky U, Fujiwara H, Suemori K, et al. HLA class I binding of HBZ determines outcome in HTLV-1 infection. *PLoS Pathog*. 2010;6:e1001117.
50. Landry S, Halin M, Lefort S, Audet B, Vaquero C, Mesnard JM, et al. Detection, characterization and regulation of antisense transcripts in HIV-1. *Retrovirology*. 2007;4:71.
51. Mayrand SM, Schwarz DA, Green WR. An alternative translational reading frame encodes an immunodominant retroviral CTL determinant expressed by an immunodeficiency-causing retrovirus. *J Immunol*. 1998;160:39–50.
52. Walsh AD, Bimber BN, Das A, Piskowski SM, Rakasz EG, Bean AT, et al. Acute phase CD8+ T lymphocytes against alternate reading frame epitopes select for rapid viral escape during SIV infection. *PLoS One*. 2013;8:e61383.
53. Maness NJ, Valentine LE, May GE, Reed J, Piskowski SM, Soma T, et al. AIDS virus-specific CD8(+) T lymphocytes against an immunodominant cryptic epitope select for viral escape. *J Exp Med*. 2007;204:2505–12.
54. Champiat S, Raposo RA, Maness NJ, Lehman JL, Purtell SE, Hasenkrug AM, et al. Influence of HAART on Alternative Reading Frame Immune Responses over the Course of HIV-1 Infection. *Plos One*. 2012;7:e39311.
55. Arpin-Andre C, Laverdure S, Barbeau B, Gross A, Mesnard JM. Construction of a reporter vector for analysis of bidirectional transcriptional activity of retrovirus LTR. *Plasmid*. 2014;74:45–51.
56. Almeida JR, Price DA, Papagno L, Arkoub L, Sauce D, Bornstein E, et al. Superior control of HIV-1 replication by CD8+ T cells is reflected by their avidity, polyfunctionality, and clonal turnover. *J Exp Med*. 2007;204:2473–85.
57. Saez-Cirion A, Bacchus C, Hocqueloux L, Avettand-Fenoel V, Girault I, Lecuroux C, et al. Post-treatment HIV-1 controllers with a long-term virological remission after the interruption of early initiated antiretroviral therapy ANRS VISCONTI Study. *PLoS Pathog*. 2013;9:e1003211.
58. Liu J, O'Brien KL, Lynch DM, Simmons NL, La Porte A, Riggs AM, et al. Immune control of an SIV challenge by a T-cell-based vaccine in rhesus monkeys. *Nature*. 2009;457:87–91.
59. Mudd PA, Martins MA, Ericson AJ, Tully DC, Power KA, Bean AT, et al. Vaccine-induced CD8+ T cells control AIDS virus replication. *Nature*. 2012;491:129–33.
60. Stephenson KE, SanMiguel A, Simmons NL, Smith K, Lewis MG, Szinger JJ, et al. Full-length HIV-1 immunogens induce greater magnitude and comparable breadth of T lymphocyte responses to conserved HIV-1 regions compared with conserved-region-only HIV-1 immunogens in rhesus monkeys. *J Virol*. 2012;86:11434–40.
61. Borthwick N, Ahmed T, Ondondo B, Hayes P, Rose A, Ebrahimsa U, et al. Vaccine-elicited Human T Cells Recognizing Conserved Protein Regions Inhibit HIV-1. *Mol Ther*. 2013;22:464–75.
62. Sunshine J, Kim M, Carlson JM, Heckerman D, Czartoski J, Migueles SA, et al. Increased sequence coverage through combined targeting of variant and conserved epitopes correlates with control of HIV replication. *J Virol*. 2013;88:1354–65.
63. Kunwar P, Hawkins N, Dinges WL, Liu Y, Gabriel EE, Swan DA, et al. Superior control of HIV-1 replication by CD8+ T cells targeting conserved epitopes: implications for HIV vaccine design. *PLoS One*. 2013;8:e64405.
64. Tang J, Wilson CM, Meleth S, Myracle A, Lobashevsky E, Mulligan MJ, et al. Host genetic profiles predict virological and immunological control of HIV-1 infection in adolescents. *AIDS*. 2002;16:2275–84.
65. Alatrakchi N, Duvivier C, Costagliola D, Samri A, Marcelin AG, Kamkamidze G, et al. Persistent low viral load on antiretroviral therapy is associated with T cell-mediated control of HIV replication. *AIDS*. 2005;19:25–33.
66. Bunce M, O'Neill CM, Barnardo MC, Krausa P, Browning MJ, Morris PJ, et al. Phototyping: comprehensive DNA typing for HLA-A, B, C, DRB1, DRB3, DRB4, DRB5 & DQB1 by PCR with 144 primer mixes utilizing sequence-specific primers (PCR-SSP). *Tissue Antigens*. 1995;46:355–67.
67. Dunbar SA. Applications of Luminex xMAP technology for rapid, high-throughput multiplexed nucleic acid detection. *Clin Chim Acta*. 2006;363:71–82.
68. Draenert R, Brander C, Yu XG, Altfeld M, Verrill CL, Feeney ME, et al. Impact of intrapeptide epitope location on CD8 T cell recognition: implications for design of overlapping peptide panels. *Aids*. 2004;18:871–6.
69. Parker KC, Bednarek MA, Coligan JE. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. *J Immunol*. 1994;152:163–75.
70. Rammensee H, Bachmann J, Emmerich NP, Bachor OA, Stevanovic S. SYFPEITHI: database for MHC ligands and peptide motifs. *Immunogenetics*. 1999;50:213–9.
71. Buus S, Lauemoller SL, Worning P, Kesmir C, Frimurer T, Corbet S, et al. Sensitive quantitative predictions of peptide-MHC binding by a 'Query by Committee' artificial neural network approach. *Tissue Antigens*. 2003;62:378–84.
72. Moris A, Nobile C, Buseyne F, Porrot F, Abastado JP, Schwartz O. DC-SIGN promotes exogenous MHC-I-restricted HIV-1 antigen presentation. *Blood*. 2004;103:2648–54.

73. Rodríguez-Plata MT, Urrutia A, Cardinaud S, Buzon MJ, Izquierdo-Useros N, Prado JG, et al. HIV-1 capture and antigen presentation by dendritic cells: enhanced viral capture does not correlate with better T cell activation. *J Immunol.* 2012;188:6036–45.
74. Streeck H, Frahm N, Walker BD. The role of IFN-gamma Elispot assay in HIV vaccine research. *Nat Protoc.* 2009;4:461–9.

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